Experimental evidence for a high rate of maternal-fetal transmission of dengue virus in the presence of antibodies in immunocompromised mice

Satoru Watanabe,[a]* Kitti Wing Ki Chan,[a] Nicole Wei Wen Tan,[a] Maharah Binte Abdul Mahid,[a] Avisha Chowdhury,[a] Kenneth Tou En Chang,[a,b] and Subhash G. Vasudevan[a,c]*

[a]Program in Emerging Infectious Diseases, Duke-NUS Medical School, 8-College Road 169857, Singapore
[b]Department of Pathology and Laboratory Medicine, KK Women's and Children's Hospital, 100 Bukit Timah Road 229899, Singapore
[c]Institute for Glycomics, Griffith University, Gold Coast Campus, Queensland 4222, Australia

Summary

Background Congenital disorders associated with prenatal vertical transmission of Zika virus (ZIKV) is established since the 2016 outbreak in the Americas. However, despite clinical reports of similar mode of transmission for other flaviviruses such as dengue virus (DENV), the phenomenon has not been experimentally explored.

Methods Pregnant AG129 mice were infected with DENV1 in the presence or absence of enhancing antibodies at different gestational time points. ZIKV was used for comparison. We quantified viral load in fetus and placentas and performed comprehensive gene expression profiling in the maternal (decidua) and fetal portion of placenta separately.

Findings We demonstrate in a laboratory experimental setting that DENV can be transmitted vertically in a gestation stage-dependent manner similar to ZIKV, and this incidence drastically increases in the presence of enhancing antibodies. Interestingly, a high rate of DENV fetal infection occurs even though the placental viral load is significantly lower than that found in ZIKV-infected dams. Comprehensive gene expression profiling revealed DENV infection modulates a variety of inflammation-associated genes comparable to ZIKV in decidua and fetal placenta in early pregnancy.

Interpretation Our findings suggest that the virus-induced modulation of host gene expression may facilitate DENV to cross the placental barrier in spite of lower viral burden compared to ZIKV. This mouse model may serve to identify the host determinants required for the vertical transmission of flaviviruses and develop appropriate countermeasures.

Introduction

Dengue is emerging as a global public health threat with an estimated 400 million human infections and several hundred thousand cases of severe dengue occurring yearly.[1] Dengue virus (DENV) infection with any of the 4 related viral serotypes (DENV1−4) causes a variety of clinical manifestations ranging from self-limiting febrile illness, known as dengue fever (DF), to the life-threatening severe diseases, such as dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS), characterized by vascular leakage, thrombocytopenia, bleeding and elevated levels of cytokines.[2−4] DHF/DSS are often associated with secondary infections where

*Corresponding authors at: Program in Emerging Infectious Diseases, Duke-NUS Medical School, 8-College Road 169857, Singapore.
E-mail addresses: satoru.watanabe@duke-nus.edu.sg (S. Watanabe), subhash.vasudevan@duke-nus.edu.sg (S.G. Vasudevan).

Research in context

Evidence before this study

The mechanism(s) that permit vertical transmission of flaviviruses is unknown. Although it has received less attention compared with Zika virus (ZIKV), prenatal vertical transmission is reported for other flaviviruses. Especially, in the case of dengue virus (DENV), it has been reported that vertical transmission rate can be as high as 18.5−22.7%, which is comparable to the case of ZIKV. However, there are so far no experimental animal models that can recapitulate and explore this phenomenon.

Added value of this study

This study shows that DENV can cross the placental barrier in an animal model, and that the rate of fetal infection drastically increases under the condition mimicking antibody-dependent enhancement of infection (ADE) in mice. This study also demonstrates that similar to ZIKV, fetal DENV infection occurs during an early stage of pregnancy when extensive cell proliferation and remodeling are underway in the placenta, and that a variety of inflammatory responses are modulated by DENV and/or ZIKV infection in placenta in a gestation-stage dependent manner in mice.

Implications of all the available evidence

This study demonstrates that gene expression pattern in placenta varies in a gestation stage-dependent manner during flavivirus infection in animal models. Detailed analysis of the molecular events associated with placentation during DENV and ZIKV infection may serve to identify the key factor(s) responsible for vertical transmission of flaviviruses and develop appropriate countermeasures.

pre-existing antibodies to a prior DENV infection form non-neturalizing complex with a currently circulating heterotypic DENV serotype, resulting in a phenomenon termed antibody-dependent enhancement (ADE) of infection.[3] At present, there are no approved antiviral drugs to prevent or treat DENV infections and only one vaccine, Dengvaxia, is commercially available in over 20 countries as a potential preventative approach. However, due to the risk of developing severe dengue in naïve individuals, the use of Dengvaxia is mostly limited to individuals who have experienced prior DENV infection.[5] Thus, the development of novel anti-dengue drugs and alternative vaccines is still an ongoing effort.

Zika virus (ZIKV) outbreak in Brazil in 2016 and the associated congenital zika syndrome (CZS)[6] has drawn attention on the risks associated with viral infection during pregnancy. ZIKV is a member of the flavivirus genus, which also includes DENV, Japanese encephalitis virus (JEV), West Nile virus (WNV), Yellow fever virus (YFV) amongst nearly 70 viruses that are

medically important. ZIKV infection is primarily associated with mild self-limiting illness, however, there is compelling evidence of its association with fetal brain and central nervous system abnormalities, such as microcephaly, which are believed to occur via direct infection of the fetus.[7] Although less well-established compared with ZIKV, it has been reported that infection by other flaviviruses, such as DENV, JEV, WNV and YFV, during pregnancy can lead to severe outcomes.[8] In the case of DENV, it has been reported previously that 2.8% of pregnant women in an endemic area had serological evidence of DENV infection during pregnancy[9] and that symptomatic dengue can be associated with adverse fetal/infant outcomes such as preterm birth and/or low birthweight,[10−14] and miscarriage or stillbirth.[12,13,15−21] It has also been reported that pregnant women infected with DENV are susceptible to increased disease severity and mortality.[18−20,22−25] Additionally, vertical transmission has been reported for DENV infection by detecting the virus genome and/or DENV-specific IgM in the fetal and cord blood at birth.[26−29] DENV infection has also been histologically detected in tissues of aborted fetuses aged 12 weeks old[28] and 29 weeks old.[30] A recent prospective cohort study revealed that DENV vertical transmission rate can be as high as 18.5−22.7%,[29] which is comparable to the 26.1% observed for ZIKV epidemic in French Guiana in 2015−2016.[31] However, despite these reports on DENV infection during pregnancy in light of the massive global footprint of dengue,[1] the issue has not received the same level of public attention as ZIKV infection probably due to the lack of any association with severe pathophysiological abnormalities such as microcephaly and other neurological deficits.

Animal models have experimentally demonstrated that ZIKV infection in pregnancy results in fetal infection and adverse pregnancy outcomes.[32] We previously showed that ZIKV infection in early pregnancy results in a high rate of fetal infection in IFN-receptor deficient mice, which can be prevented by therapeutic neutralizing antibodies (Abs)[33] or antivirals.[34] Here, we show that DENV can also cross the placental barrier in an animal model, and that the rate of fetal infection drastically increases under ADE condition in IFN receptors deficient mice (AG129 mice). We also show that similar to ZIKV, a high rate of vertical transmission of DENV occurs during early stages of pregnancy when extensive cell proliferation and differentiation are underway in the placenta. Comprehensive gene expression profiling reveals that gene expression pattern is different between maternal decidua and fetal placenta at various stages of pregnancy. A variety of inflammatory responses are commonly, but in part specifically, modulated by DENV and/or ZIKV infection in early pregnancy. The mechanism(s) that permit vertical transmission of flaviviruses is unknown and few studies have attempted to elucidate the mechanism from the perspective of host molecular

events in placenta. Therefore, our study may provide a pathway to understand the mechanisms of transplacental infection of flaviviruses and develop therapeutics for its prevention.

Methods

Cells and viruses
BHK-21 cells (baby hamster kidney fibroblast cells, ATCC: CCL-10) and C6/36 (*Aedes albopictus* cells, ATCC: CRL-1660) were cultured as described previously.[35] DENV1−2402 (EDEN1, GenBank accession EU081230.1) was obtained from the Early Dengue infection and outcome (EDEN) study in Singapore[36] and passaged 6 times in C6/36 cells prior to use in this study. ZIKV Paraiba 01/2015 (Brazilian strain) was a gift from Evandro Chagas Institute and passaged 2 times in C6/36 cells prior to use in this study. All virus strains were grown in C6/36 cells and the supernatants were stored at −80 °C after filtration through a 0.45 μm membrane. Virus titer was determined by standard plaque assay on BHK-21 cells. 4G2 antibody (Ab) (mouse IgG2a, anti-E of all DENV serotypes) was purified from supernatant of cultured hybridoma (ATCC: HB-112) as described previously.[35]

Animal studies
All animal experiments (protocol 2016/SHS/1197; 2021/SHS1651) were approved by the Institutional Animal Care and Use Committee at Singapore Health Services and conformed to the National Institutes of Health (NIH) guidelines and public law.

Sv/129 mice deficient in type I/II IFN receptors (AG129), purchased from B&K Universal (UK), were housed in the BSL-2 animal facility at Duke-NUS, Singapore. Eight to 12 week-old mice were used for all experiments.

DENV1 or ZIKV infection in non-pregnant mice
Eight to 10 weeks old adult female AG129 mice were inoculated intravenously (iv) with 2×10^7 pfu of DENV1 or 1×10^3 pfu of ZIKV Paraiba strain. Blood was collected on days 1−8 post-infection (pi) from facial vein and the serum samples were used to measure viral copy number. Mouse survival rate was monitored until day 12.

DENV1 or ZIKV infection in pregnant mice
Eight to 12 weeks old female AG129 mice were used to obtain pregnant mice. Female mice were housed with adult male AG129 mice for 3.5 days. The female mice were then inoculated iv with 2×10^7 pfu of DENV1 either on day 7.5 post-mating (corresponding to E4−7.5) or day 11.5 post-mating (corresponding to E8−11.5). Mice that acquired infection in the E4−7.5 period were

sacrificed on day 7 pi (E11−14.5), and mice that were infected in the E8−11.5 period were sacrificed on day 3 (E11−14.5), 5 (E13−16.5) or 7 (E15−18.5) pi. In separate experiments, female AG129 mice were housed with adult male AG129 mice for 1.5 days and inoculated iv with 2×10^7 pfu of DENV1 or 1×10^3 pfu of ZIKV Paraiba on day 7.5 post-mating (E6−7.5), day 9.5 post-mating (E8−9.5), or day 13.5 post-mating (E12−13.5). The copulatory plug formation[37] was checked every morning for 2 days. For the induction of ADE infection of DENV, mice were injected iv with 50 μg of 4G2 Ab 4 h prior to DENV infection. Mice that acquired infection in the E6−7.5 period were sacrificed on day 3 pi (E9−10.5) or day 5 pi (E11−12.5), and mice that were infected in the E8−9.5 period and the E12−13.5 period were sacrificed on day 5 (E13−14.5 and E17−18.5, respectively). All mice were euthanized by CO_2 inhalation and perfused with PBS after blood collection from the postcaval vein. Fetuses were collected and washed with PBS three times on 24- or 48-well plates to avoid contamination of maternal blood-derived viruses. In some experiments, placentas were separated into the fetal part and the maternal decidua part[38] to analyze individually. Fetuses and tissues were snap frozen in liquid nitrogen until use.

Quantification of viral genome copies and host gene expression by quantitative real-time RT-PCR
Serum viral RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. Frozen tissues were homogenized by TissueLyser (Qiagen) with 100−1000 μl PBS depending on the tissue size, and the supernatants (10−20 μl) after centrifugation were used for RNA extraction using Trizol (Thermo Fisher Scientific). The RNA concentration was measured by Nanodrop (Thermo Fisher Scientific) and adjusted to 20 ng/μl in distilled water. Eighty ng of RNA was used to measure viral RNA and host gene expression levels by real-time PCR.

Real-time RT-PCR to measure viral RNA was carried out in Bio-Rad Real-time thermal cycler CFX96 by the use of qScript One-Step qRT-PCR kit (Quanta) according to the manufacturer's instructions. Primers and TaqMan probes targeting for DENV1 and ZIKV (Table S1), and RNA products used for the standard curve for quantification of viral genome copy were described in our previous study.[39]

RNA extracted from placenta samples was further subjected to real-time RT-PCR quantification for the host genes expression by iTaq Universal SYBR green one-step kit according to the manufacturer's instructions (Bio-Rad). Primers targeting β-actin,[40] caspase-1, caspase-11/4, caspase-6, caspase-7, caspase-12[41] and caspase-8[42] were described in Table S1. The gene expression was normalized to β-actin expression and presented as relative values calculated by $2^{-\Delta\Delta Ct} * 10,000$.

Determination of infectious virus titer by plaque assay

Supernatants of fetus and placenta homogenates were diluted 2−10-fold with culture media and subjected to standard plaque assay to measure the infectious virus titer using BHK-21 cells as described previouly.[43]

Gene expression profiling in placenta samples by NanoString technologies

Gene expression profiling using NanoString nCounter gene expression assay was performed as described previously.[44] Briefly, RNA used for the NanoString assay was extracted from the homogenates of fetal placenta and decidua using RNeasy Micro Kit (Qiagen) following manufacturer's instructions. The quality and quantity of the RNA extracted were assessed by Bioanalyzer (Agilent Technologies) and Quant-iT RiboGreen RNA assay kit (Life Technologies), respectively. 100 ng of total RNA was subjected to inflammatory genes profiling using a customized nCounter® Mouse Inflammation V2 codeset (NanoString Technologies) according to manufacturer's instructions. The add-on genes in the nCounter® Mouse Inflammation V2 codeset can be found in Table S2. The gene expression profile obtained from nCounter® was analyzed using nSolver 3.0. Gene counts are normalized to the panel's internal housekeeping genes and expressed as $\log_2$ (fold change) with respect to uninfected control placentas with heatmaps generated using online Morpheus software (https://software.broadinstitute.org/morpheus).

Statistical analysis

Graphs were generated using GraphPad Prism v5.0 software or Microsoft Excel. Significant differences between data groups were determined by 2-tailed Student t-test analysis (https://www.socscistatistics.com/tests/studentttest/). Significant differences of the fetal infection rate between data groups were determined by Fisher's exact test (https://www.socscistatistics.com/tests/fisher/default2.aspx). P value less than 0.05 was considered significant.

Role of the funders

Results

Non-lethal DENV1 infection during pregnancy induces up to 25% fetal infection in AG129 mice

DENV has been shown to be more infectious in AG129 mice (lacking IFN-α/β and γ receptors) compared with immunocompetent mice or A129 mice (lacking IFN-α/β receptor).[45] We previously showed that high titer inoculum ($>10^7$ pfu) of DENV1 clinical strain EDEN1 (GenBank accession: EU081230.1) can induce high levels of serum viral RNA (viremia) in AG129 mice.[46] Therefore, we selected this mouse strain to examine the potential for DENV to be disseminated through transplacental vertical transmission. We first established that intravenous (iv) infection with 2×10^7 pfu of DENV1 (EDEN1) strain induced viremia that reached $> 10^7$ copies/ml on day 2−3 post-infection (pi) (Figure 1a) without mortality (Figure 1b) in non-pregnant female AG129 mice. On the other hand, ZIKV has been shown to be more infectious in immunocompromised mice and inoculation with 1×10^3 pfu of ZIKV strains in A129 was shown to induce a high rate of fetal infection in our previous studies.[34,39,47] As shown in Figure 1a and 1b, ZIKV-Paraiba (Brazilian strain), that was used as a comparator in this study, caused 100% mortality around day 10 pi accompanied with higher levels of viremia than DENV1 when inoculated with 1×10^3 pfu. These infection conditions were adopted for the experiments using pregnant AG 129 mice.

In both humans as well as mice, fetal ZIKV infection occurs during the early stage of pregnancy.[34,48−51] Therefore, we initially inoculated DENV1 in pregnant mice at two different embryonic stages; on day 7.5 post-mating (Figure 1c-i) or day 11.5 post-mating (Figure 1c-ii). Since the estrous cycle in female mice is typically 4 days long, mating for 3.5 days can achieve a high rate of pregnancy required to examine an overall trend of vertical transmission. Therefore, the infection days corresponded to embryonic days 4−7.5 (E4−7.5) (Figure 1c-i) and E8−11.5 (Figure 1c-ii), respectively. Pregnant mice that acquired infection at the E4−7.5 stage were sacrificed on day 7 pi (E11−14.5), while mice that acquired infection at the E8.5−12 stage were sacrificed on day 3 (E11−14.5), 5 (E13−16.5) or 7 pi (E15−18.5) in order to harvest fetuses and placentas (Figure 1c). The fetus and whole placenta samples were homogenized with PBS and RNA was extracted from the supernatants to measure the tissue viral load by realtime RT-PCR.[35] To avoid contamination of maternal blood-derived viruses, harvested fetuses were washed with PBS three times before being snap frozen. Virus genome was detected in all placentas examined and the genome copies showed an increasing trend from day 3 to day 7 pi (Figure 1d). We found that 23% (5/22) of the fetuses were positive for DENV on day 7 pi in dams that acquired infection at E4−7.5, whereas the DENV positive rates in dams that acquired infection at E8−11.5 were 9% (2/23) on day 3 pi, 23% (6/26) on day 5 pi, or 4% (1/26) on day 7 pi, respectively (Figure 1e). Collectively, this data indicates that up to 25% fetuses can be infected by DENV when dams acquire infection within the range of E4−11.5. However, it also should be noted that a rate of fetal infection is different among the
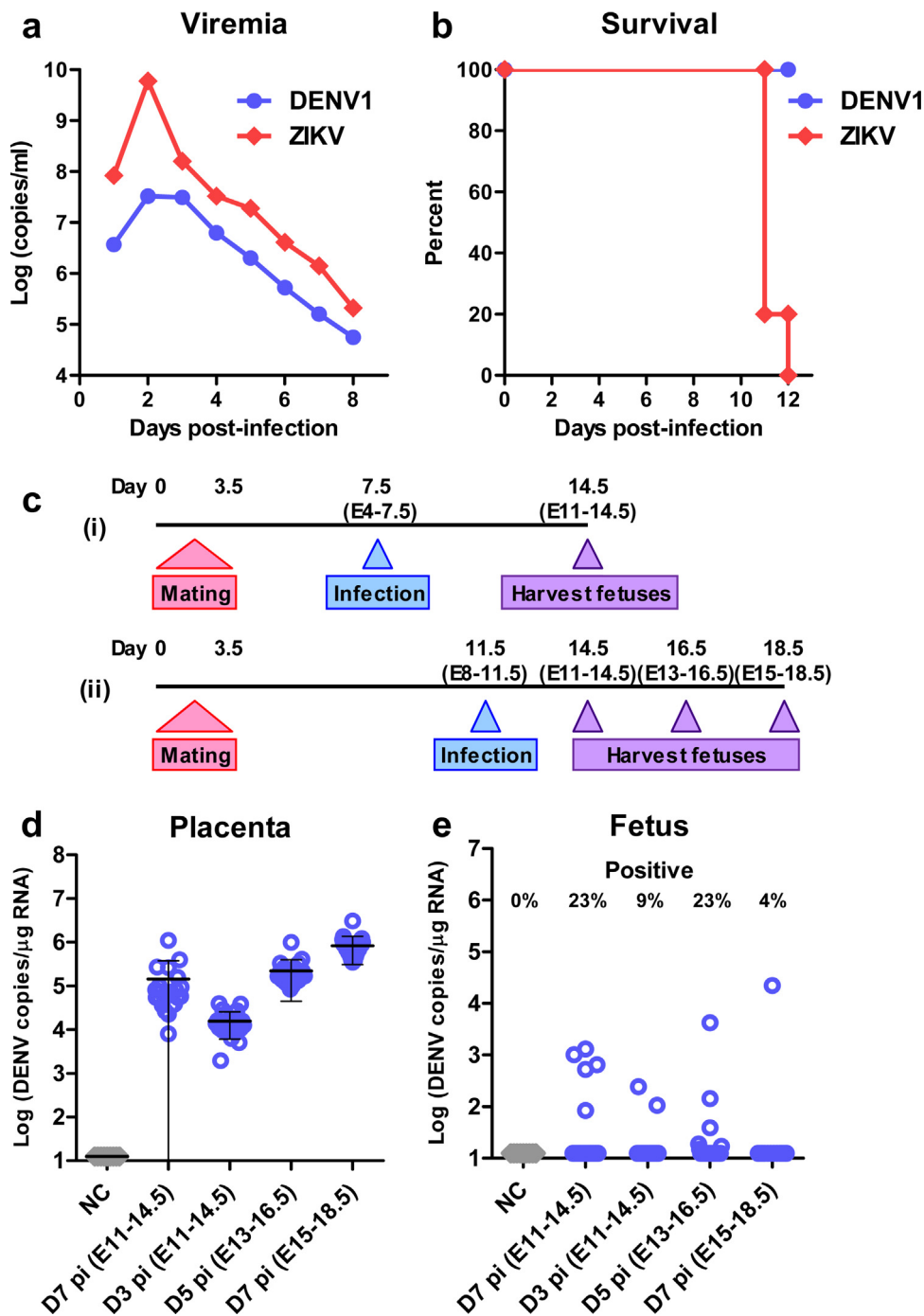
Figure 1. DENV1 pathogenesis in pregnant mice. (a and b) Non-pregnant adult female AG129 mice were inoculated iv with DENV1 (EDEN1; 2×10^7 pfu) or ZIKV (Paraiba; 1×10^3 pfu). Blood samples were collected on days 1- 8 post-infection (pi) and mixed serum from each group were subjected to real-time RT-PCR to obtain the average viral genome copy numbers (a). Mouse survival rate was monitored until day 12 pi (b). The number of mice per group is 5. (c−e) Eight to 12 weeks old female AG129 mice were housed with adult male AG129 mice for 3.5 days. The female mice were inoculated iv with 2×10^7 pfu of EDEN1 on day 7.5 post-mating (corresponding to E4−7.5) or day 11.5 post-mating (corresponding to E8−11.5). Mice that acquired infection at E4−7.5 were sacrificed on day 7 pi (E11−14.5), and mice that acquired infection at E8−11.5 were sacrificed on day 3 (E11−14.5), 5 (E13−16.5) or 7 (E15−18.5) pi (c). Whole placentas (d) and fetuses (e) were collected to measure viral load by real-time RT-PCR. The limit of PCR detection is 12.5 copies/μg RNA. Placental viral load was plotted with the average results with standard deviations. NC indicates uninfected control group harvested at E11−14.5.

individual dams and a trend that younger fetuses, which are morphologically distinguishable, are more likely to acquire infection at each time point of harvest was observed (Figure S1). The dams that bore DENV-positive fetuses at a rate of >20% are marked with asterisks in Figure S1.

DENV infection in the presence of enhancing antibodies drastically increases the risk of fetal infection and mortality in mice

Having established the conditions for significant fetal infection when dams become infected, we next explored the impact of severe dengue associated with ADE infection, a phenomenon that is well established in experimental models and supported epidemiologically in the context of DENV patients who have experienced secondary heterotypic DENV infection[52,53] or who have had ZIKV infection prior to DENV.[54] Next, to avoid a large difference in the homogeneity, mice were mated for a shorter period of time (1.5 days long) and on day 9.5 post-mating (E8−9.5), they were injected iv with $50\mu g$ of 4G2 antibody that binds to the envelop (E) protein of flaviviruses 4 h prior to infection with DENV1 to mimic ADE infection[46] (Figure 2a). The mice that received 4G2 Abs showed more obvious signs of infection such as ruffled fur,[35] however, they did not succumb to infection. Mice were sacrificed on day 4 or 5 pi, when the effect of ADE is prominently observed in tissues,[35,55] in order to harvest fetuses, placentas and various maternal tissues (Figure 2a). Among the tissues examined, a significant increase in viral load was observed in liver (16.9-fold; $P = 0.030$ on day 4 and 3.6-fold; $P = 0.001$ on day 5) and small (S) intestine (2.6-fold; $P = 0.011$ on day 4 and 2.4-fold; $P = 0.002$ on day 5) (Figure S2), which was similarly observed in AG129 mice infected with DENV2 with enhancing Abs.[35,55] Under this ADE condition, placental viral load was observed to be increased by 1.7-fold ($P<0.001$) on day 4 pi and 2.4-fold ($P = 0.002$) on day 5 pi (Figure 2b), suggesting that placenta is one of the target tissues for ADE of DENV in mice. Notably, the rate of DENV-positive fetus drastically increased under the conditions mimicking ADE with 4G2 Ab on both day 4 pi [Virus control (VC): 17.2% (5/29) vs ADE: 63.0% (17/27), $P = 0.001$] and day 5 pi [VC: 17.6% (3/17) vs ADE: 63.6% (21/33), $P = 0.003$] (Figure 2c). Higher rate of fetal death mirrored the increased infectivity that was observed in dams infected after 4G2 Ab treatment on day 4 pi (VC: 3.4% vs ADE: 18.5%) and day 5 pi (VC: 11.8% vs ADE: 48.5%) (Figure 2d). Intriguingly, 2.4-fold increase in placental viral load in the presence of Ab (Figure 2b) resulted in more than 30% increase in fetal mortality on day 5 pi (Figure 2d). DENV1 was detected in 91.7% (22/24) of the dead fetuses that could be clearly identified by the abnormal shape/bloodless color (Figures 2d and S3), suggesting that fetal death occurred as a direct

consequence of DENV infection. Taken together, the risk of fetal infection and death is clearly increased in the presence of enhancing Ab in our mouse model.

High rate of DENV fetal infection can be established even though the placental viral load is lower than that of ZIKV

Parallel studies with ZIKV-Paraiba strain in pregnant AG129 mice for comparison showed that 56.7% (17/30) of the fetuses were positive on day 5 pi, which is comparable to the rate of DENV fetal infection under ADE condition ($P = 0.614$) (Figure 2c). Surprisingly, however, placental viral load in DENV infection was found to be 21.7-fold lower than ZIKV infection ($P<0.001$) (Figure 2b). Plaque assay to detect infectious virus in the same placenta samples showed that the DENV titer in the presence of 4G2 Ab (6.3×10^1 PFU) was 65.4-fold lower than the ZIKV titer (4.1×10^3 PFU) (Figure S4). These results suggests that viral burden in placenta is perhaps not the only determinant that leads to vertical transmission of virus. In addition, infectious virus particles could be detected in 6% (1/17), 27.3% (9/33) and 33.3% (10/30) of the fetuses in dams with DENV (-Ab), DENV (+Ab) and ZIKV infection, respectively (Figure S4), which corroborates that the infection occurred in these fetuses. ZIKV infection induced a lower rate of fetal death (10.0%) compared with DENV-ADE (48.5%) infection on day 5 pi (Figure 2d). Among the infected fetuses, 2 out of 3 (66.7%) were found dead in DENV infection without Ab and 16 out of 21 (76.2%) were dead in DENV infection with 4G2 Ab on day 5 pi, whereas 3 out of 17 (17.6%) were found dead in ZIKV infection on day 5 pi (data not shown), also suggesting that this DENV strain may be more pathogenic in murine fetuses than ZIKV in the present experimental conditions.

DENV infection at late pregnancy does not permit fetal infection in mice

Since younger fetuses seemed to be more likely to acquire DENV infection when the dams are infected at E8−11.5 (Figure S1), we further corroborated this observation by infecting virus at the late pregnancy stage on day 13.5 post-mating (E12−13.5), when the placenta becomes morphologically and functionally mature,[56] and collecting the fetuses and whole placentas on day 5 pi (E17−18.5) (Figure 2e). Strikingly, none of the fetuses were positive for DENV even in the presence of the enhancing Ab (Figure 2g). Lack of viral load enhancement by 4G2 Ab in placenta (Figure 2f) suggests that ADE infection condition in placenta may decrease at the late stage of pregnancy. Significant reduction in the rate of fetal infection was also observed in ZIKV-infected mice (4/26: 15.4%) (Figure 2g) at the late stage of pregnancy as was also shown previously in mouse
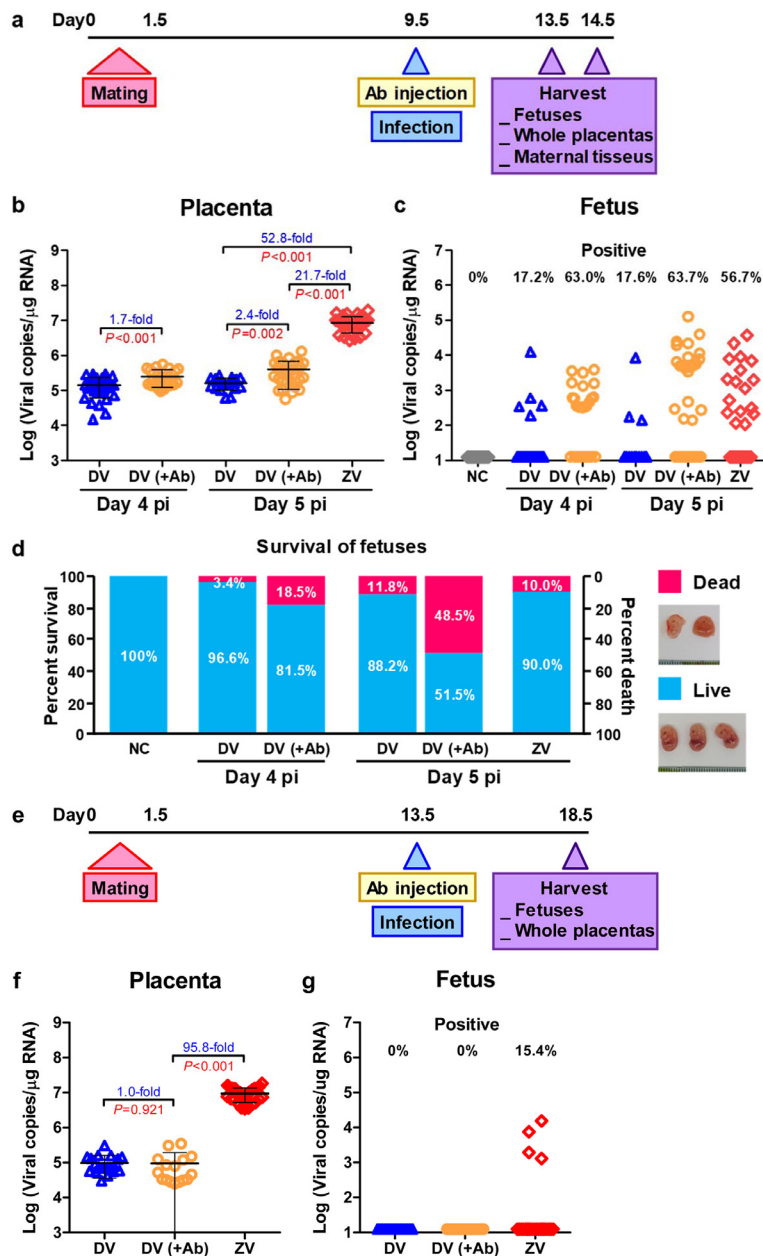
Figure 2. DENV1 pathogenesis in the presence or absence of enhancing Ab in pregnant mice, and its comparison with ZIKV pathogenesis. (a-d) Eight to 12 weeks old female AG129 mice were housed with adult male AG129 mice for 1.5 days and inoculated iv with 2×107 pfu of EDEN1 in the absence (DV) or presence [DV (+Ab)] of 4G2 Ab, or 1×10^3 pfu of ZIKV Paraiba (ZV) on day 9.5 post-mating (E8−9.5). 50μg of 4G2 were injected iv 4 h prior to DENV infection. DENV-1 infected mice were sacrificed on day 4 (E12−13.5) or 5 (E13−14.5) pi, and ZIKV-infected mice were sacrificed on day 5 (E13−14.5) pi (a). Viral load in whole placentas (b) and fetuses (c) were measured by real-time RT-PCR and plotted with the average results with standard deviations for placentas. Percentages of live/dead fetuses, that were visually identifiable, are shown in d. The representatives of live/dead fetuses are displayed on the pictures. NC indicates uninfected control group harvested at E12−13.5. (e−g) Eight to 12 weeks old female AG129 mice were housed with adult male AG129 mice for 1.5 days and inoculated iv with 2×10^7 pfu of EDEN1 in the absence (DV) or presence [DV (+Ab)] of 4G2 Ab, or 1×10^3 pfu of ZIKV Paraiba on day 13.5 post-mating (E12−13.5). 50μg of 4G2 were injected iv 4 h prior to DENV infection. DENV- and ZIKV-infected mice were sacrificed on day 5 pi (E17−18.5) (e). Viral load in placentas (f) and fetuses (g) was measured by real-time RT-PCR and plotted with the average results with standard deviations for placentas. The limit of PCR detection is 12.5 copies/μg RNA, and samples with >12.5 copies/μg were counted as positive. The numbers of samples of data groups are indicated in the text. Statistical differences of viral load between data groups were analyzed using 2-tailed Student t-test. Significant differences of the fetal infection rate between data groups were determined by Fisher's exact test.

models.[34,49,50] These results indicate that the vertical transmission of DENV predominantly occurs at early pregnancy similar to ZIKV in mice.

DENV preferably infects the maternal decidua under the condition of ADE, while a fetal portion of the placenta is a dominant site of ZIKV infection

Placenta consists of the embryo-derived fetal tissue and uterine-derived maternal decidua. The fetal site is mainly composed of Hofbauer cells and various types of trophoblast, whereas the maternal decidua mainly consists of the decidual stroma cells and a variety of maternal immune cells such as macrophages, dendritic cells (DCs) and NK cells.[57] Since DENV infection in the presence of 4G2 Ab induced comparable levels of fetal infection to ZIKV infection (Figure 2c) despite the significant difference in placental viral load (Figure 2b), we next examined the detailed pathogenesis in the fetal and maternal portions of the placenta of these 2 infections. Mice were mated for 1.5 days and infected with DENV1 in the presence of 4G2 or ZIKV on day 9.5 post-mating (E8−9.5) (Figure 3a) as was done in the previous experiment (Figure 2a). Fetuses and placentas were harvested on day 5 pi and the placentas were further separated into the fetal and maternal portions (Figure 3a). The rate of fetal infection was comparable between DENV (8/17: 47.1%) and ZIKV (7/12: 58.3%) ($P = 0.710$) (Figure 3b). Interestingly, the viral load of DENV in decidua (1.5×10^6 copies/μg RNA) was 5.8-fold higher than that in fetal placenta (2.5×10^5 copies/μg RNA) ($P = 0.007$) (Figure 3i), whereas the viral load of ZIKV in fetal placenta (1.8×10^7 copies/μg RNA) was 3.3-fold higher than that in decidua (5.5×10^6 copies/μg RNA) ($P<0.001$) (Figure 3i). Consequently, the viral load in ZIKV infection was 72.4-fold higher than DENV infection in the fetal portion of placenta ($P<0.001$) (Figure 3c) and only 3.8-fold higher in decidua although the difference is still statistically significant ($P = 0.001$) (Figure 3d).

Since the physiological environment of placenta undergoes dynamic changes during pregnancy, we further examined the DENV and ZIKV pathogenesis at an earlier stage of pregnancy. Pregnant mice were infected with DENV1 in the presence of 4G2 or ZIKV on day 7.5 post-mating (E6−7.5) and fetuses, fetal placenta and decidua were harvested on day 3 pi (E9−10.5) (Figure 3e). The rate of fetal ZIKV infection increased to 100% (9/9) (Figure 3f) even though the viral load in fetal placenta and decidua is 4.7-fold and 2.0-fold lower than that on day 5 pi (Figure 3c and d), respectively, indicating that the placenta in earlier pregnancy is more permissive for ZIKV transmission regardless of viral burden. On the other hand, the rate of fetal DENV infection was only 27.3% (3/11) even in the presence of enhancing Ab (Figure 3f). At this stage, the viral load of DENV in decidua (8.3×10^4 copies/μg RNA) was 1.7-fold higher than that in fetal placenta (4.8×10^4 copies/μg RNA) ($P = 0.034$) (Figure 3i), whereas the viral load of ZIKV in fetal placenta (3.9×10^6 copies/μg RNA) was 1.5-fold higher than that in decidua (2.7×10^6 copies/μg RNA) ($P = 0.012$) (Figure 3i). Consequently, the viral load in ZIKV infection was 81.4-fold higher than DENV1 infection in the fetal placenta ($P<0.001$) (Figure 3g) and 32.5-fold higher in decidua ($P<0.001$) (Figure 3h). Thus, the viral load in the decidua seems to be associated with the difference in the rate of fetal infection between DENV and ZIKV.

One possible reason for the low rate of fetal DENV infection is that it might take more than 3 days to see clearly the effect of ADE.[35] Therefore, we further collected samples from DENV1-infected mice on day 5 pi (E11−12.5) (Figure 3e). The rate of DENV-positive fetus increased to 53.3% (8/15), suggesting that the vertical transmission of DENV under ADE condition occurs later than that of ZIKV. Nevertheless, the positive rate for DENV is similar to that of fetuses at E13−14.5 (47.1%; Figure 3b), indicating that embryonic stage-dependent fetal DENV infection is not as clearly inducible as ZIKV at this pregnancy stage.

Gene expression pattern in placenta varies depending on gestational age

Virus infection induces various host biological reactions and one possible route of viral transmission during pregnancy is that placental infection may lead to breakdown of the placental barrier by inducing inflammation or apoptotic cell death, as suggested by mouse models of ZIKV infection.[58,59] We therefore comprehensively analyzed the gene expression in placentas using NanoString nCounter gene expression assay. To minimize the differences in embryonic age among the samples, we selected placenta samples obtained from dams that had carried a copulation plug on the first day.[37] RNA extracts (100 ng) of fetal placenta and decidua collected at E14.5 ($n = 4$) (Figure 3a) and at E10.5 ($n = 4$) (Figure 3e) together with uninfected control harvested on each corresponding day ($n = 4$) were subjected to the gene expression profiling using nCounter® Mouse Inflammation V2 codeset with add-on genes (Table S2) that comprises 278 genes related to inflammation, innate and adaptive immune responses and apoptosis Figure 4. shows the overall profile of mRNA copy numbers per gene in fetal placenta and decidua from uninfected control (NC), DENV- and ZIKV-infected dams. A number of genes were found to be transcriptionally active in the fetal placentas regardless of infection at both E10.5 (Figure 4a) and E14.5 (Figure 4c). Interestingly, however, most of the genes including housekeeping genes were found to be transcriptionally silent in decidua at E14.5 (Figure 4d), although various genes in decidua were transcriptionally active at E10.5 (Figure 4b) similar to the fetal placentas (Figure 4a).
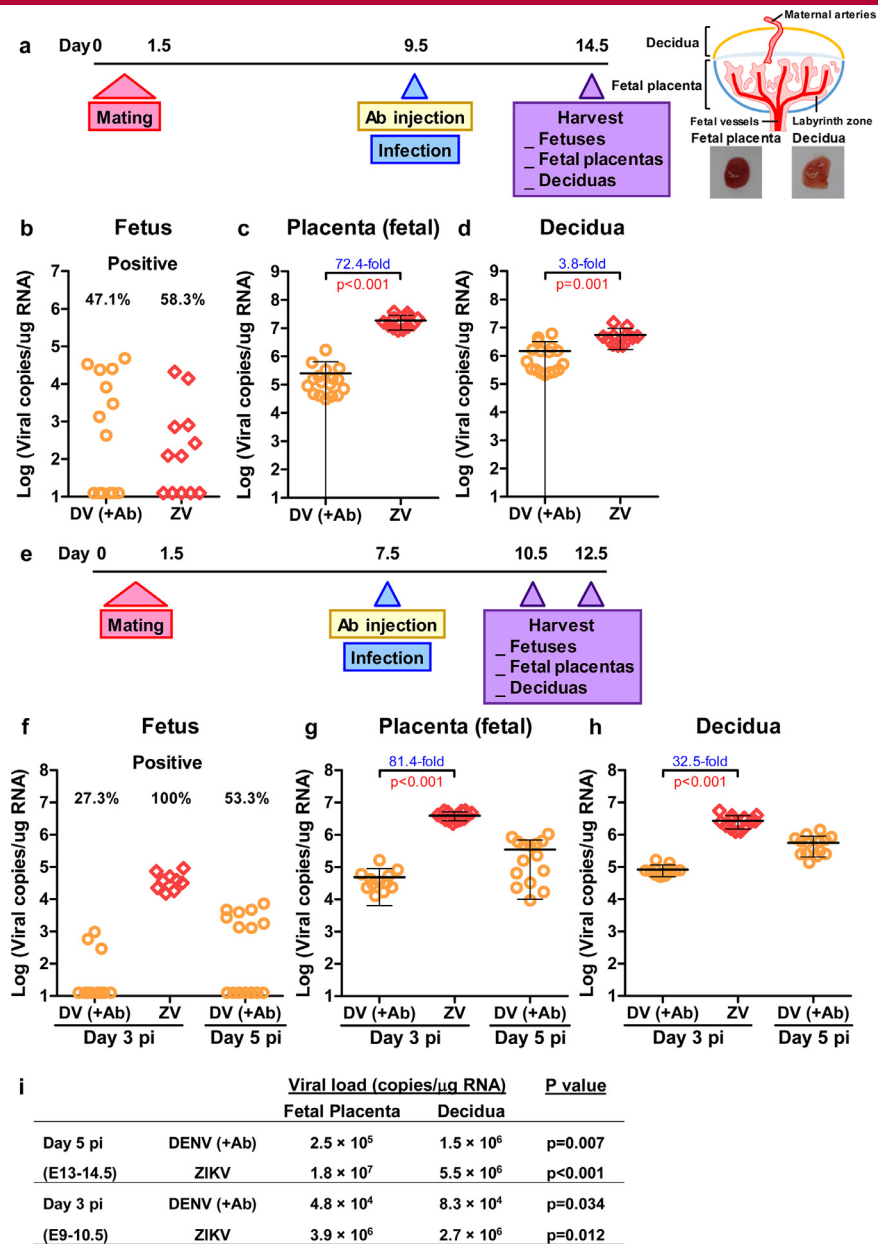
Figure 3. DENV1 and ZIKV infection in a fetal portion of placenta and maternal decidua. (a−d) Eight to 12 weeks old female AG129 mice were housed with adult male AG129 mice for 1.5 days and inoculated iv with 2×10^7 pfu of EDEN1 in the presence of 4G2 Ab [DV (+Ab)] or 1×10^3 pfu of ZIKV Paraiba (ZV) on day 9.5 post-mating (E8−9.5). 50μg of 4G2 were injected iv 4 h prior to DENV infection. DENV- and ZIKV-infected mice were sacrificed on day 5 pi (E13−14.5) (a). Placentas were separated into a fetal portion and maternal decidua as displayed on the pictures. A simplified diagram of mature mouse placenta is also shown (a). Viral load in fetuses (b), fetal placentas (c) and deciduas (d) was measured by real-time RT-PCR and plotted with the average results with standard deviations for placentas. (e−h) Eight to 12 weeks old female AG129 mice were housed with adult male AG129 mice for 1.5 days and inoculated iv with 2×10^7 pfu of EDEN1 in the presence of 4G2 Ab [DV (+Ab)] or 1×10^3 pfu of ZIKV Paraiba (ZV) on day 7.5 post-mating (E6−7.5). 50μg of 4G2 were injected iv 4 h prior to DENV infection. DENV- and ZIKV-infected mice were sacrificed on day 3 (E9−10.5) or day 5 (E11−12.5) pi (a). Viral load in fetuses (b), fetal placentas (c) and deciduas (d) was measured by real-time RT-PCR and plotted with the average results with standard deviations for placentas. The limit of PCR detection is 12.5 copies/μg RNA, and samples with >12.5 copies/μg were counted as positive. (i) Comparison of viral load between fetal placenta and decidua and its P values on day 5 pi (c and d) and day 3 pi (g and h). The numbers of samples of data groups are indicated in the text. Statistical differences of viral load between data groups were analyzed using 2-tailed Student *t*-test. Significant differences of the fetal infection rate between data groups were determined by Fisher's exact test.
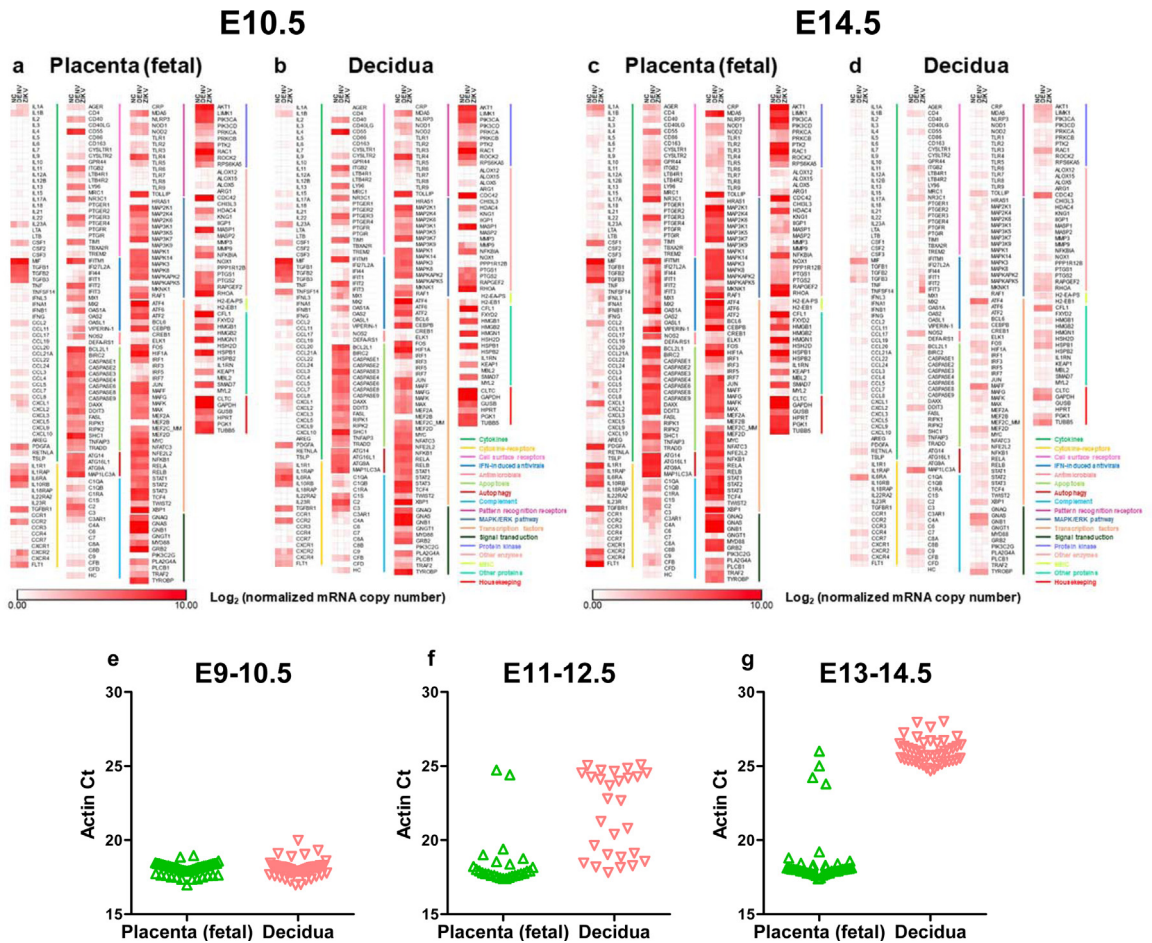
Figure 4. Overall profile of mRNA copy numbers of inflammatory genes in fetal placenta and decidua analyzed by NanoString nCounter gene expression assay. (a−d) Placenta samples obtained from dams that had carried a copulation plug on the first day morning were selected for gene expression profiling by NanoString assay. RNA was extracted from 12 fetal placentas and deciduas harvested from uninfected control mice ($n = 4$), DENV1-infected mice (+Ab) ($n = 4$), and ZIKV-infected mice ($n = 4$) at E10.5 (a and b) and E14.5 (c and d). 100μg RNA per sample was subjected to gene expression analysis using nCounter® Mouse Inflammation V2 codeset with add-on genes (Table S2). Messenger RNA copy numbers of 278 genes were normalized to that of panel's internal housekeeping genes (6 genes) and the average RNA copy numbers of 4 samples per group were expressed as log₂ with heatmap. The maximum color density of the heatmap is 1024 (log₂10) RNA copies. (e-g) All fetal placenta and decidua samples harvested on E9−10.5 ($n = 50$) (e), E11−12.5 ($n = 28$) (f) and E13−14.5 ($n = 43$) (g) were subjected to real-time RT-PCR for β-actin to see the kinetics of gene expression determined by the cycle threshold (Ct) values.

This indicates that the gene activity decreases in the decidua in a restricted manner during the process of prenatal development. In order to corroborate this observation, the kinetics of actin expression was examined by realtime RT-PCR using the placenta samples collected at E9−10.5, E11−12.5 and E13−14.5 (Figure 3a and e). Threshold Cycle (Ct) values of actin were mostly < 20 in fetal placenta all the time (Figure 4e−g), whereas actin Ct values in decidua were < 20 at E9−10.5 (Figure 4e) and > 25 at E13−14.5 (Figure 4g), which correlates with the gene expression profile obtained by NanoString assay (Figure 4a−d). In addition, varied actin Ct values ranging from 17 to 27 were

detected in deciduas at E11−12.5 (Figure 4f), indicating that the gene expression activity in decidua drastically changes during E11−13 in mice.

DENV and ZIKV infection commonly, and in part specifically, modulate host immune and inflammatory responses
In decidua at E14.5, only limited genes such as IFIT were modulated by virus infection (Figure 4d). The overall gene expression profiles in response to DENV or ZIKV infection in fetal placenta at E14.5 and fetal placenta and decidua at E10.5 are displayed as fold change

with respect to uninfected control placentas in Figures S6, S7 and S8, respectively. Of the 278 profiled genes, we found that 16 genes were significantly upregulated by both DENV and ZIKV infection, while 8 and 15 genes were uniquely upregulated by either DENV or ZIKV infection, respectively, in fetal placenta at E14.5 (Figure 5a). The most prominent upregulation was found in the genes encoding IFN-related proteins such as IFITs, MX2, OASs, Viperin-1, IFNs, IRF7 and STATs, which are predominantly induced by ZIKV (Figures S6 and 5a), as previously shown in various types of cells.[60] On the other hand, the expressions of complements such as C4A, C6 and CFB were characteristics of DENV infection in fetal placentas at E14.5 (Figures S6 and 5a).

In fetal placenta at E10.5, total 32 genes were significantly upregulated by DENV and/or ZIKV infection, and IFN-related genes such as IFITs, OASs, IRF7 and STATs were predominantly upregulated by ZIKV (Figures S7 and 5b) similar to E14.5 (Figure 5a). However, the gene profile at this stage is characterized by a significant downregulation of a number of genes (total 31 genes), which includes apoptosis-related genes (CASPASEs and SHC1), complements (C1s), pattern recognition receptors (TLR3 and 4) and transcription factors (NFKB1 and STAT3), by DENV and/or ZIKV infection (Figure 5b). In addition, several genes such as IFITM1 and MMP9 were oppositely regulated by infection between E14.5 (Figure 5a) and E10.5 (Figure 5b). The take home message is that the host immune responses to virus infection vary depending on the gestational age in placentas. In decidua at E10.5, 18 genes were found to be commonly upregulated by DENV and ZIKV infection, and a higher number of genes were upregulated by DENV infection (18 genes) in a biased manner compared with ZIKV infection (8 genes) (Figure 5c) in spite of 32.5-fold lower DENV viral load (Figure 3h). Interestingly, a number of the apoptosis-related genes (CASPASEs, BCL2L1, DAXX and DDIT3) were found to be upregulated predominantly by DENV infection in decidua (Figures S8 and 5c). It is also notable that many transcription factors such as BCL6, CEBPB, FOS, IRF1, MAFF and MEFs were upregulated by DENV/ZIKV infections more prominently than fetal placenta (Figures S8 and 5c), suggesting that decidua is more transcriptionally responsive to infections than fetal placenta at this stage, even though the gene expression in decidua appears to be silent and unresponsive to infection at the later stage of pregnancy (Figure 4d).

In order to validate the gene expression profiles obtained by NanoString assay, the expressions of several Caspases were further examined by realtime RT-RCR using increased number of decidua samples collected at E9−10.5. Caspase-1 (Figure 6a) and Caspase-11/4 (Figure 6b) were upregulated by both DENV and ZIKV infection similarly to the NanoString data (Figure 5c). The expression of Caspase-8 was also upregulated by DENV/ZIKV infection (Figure 6c), whereas Caspase-6
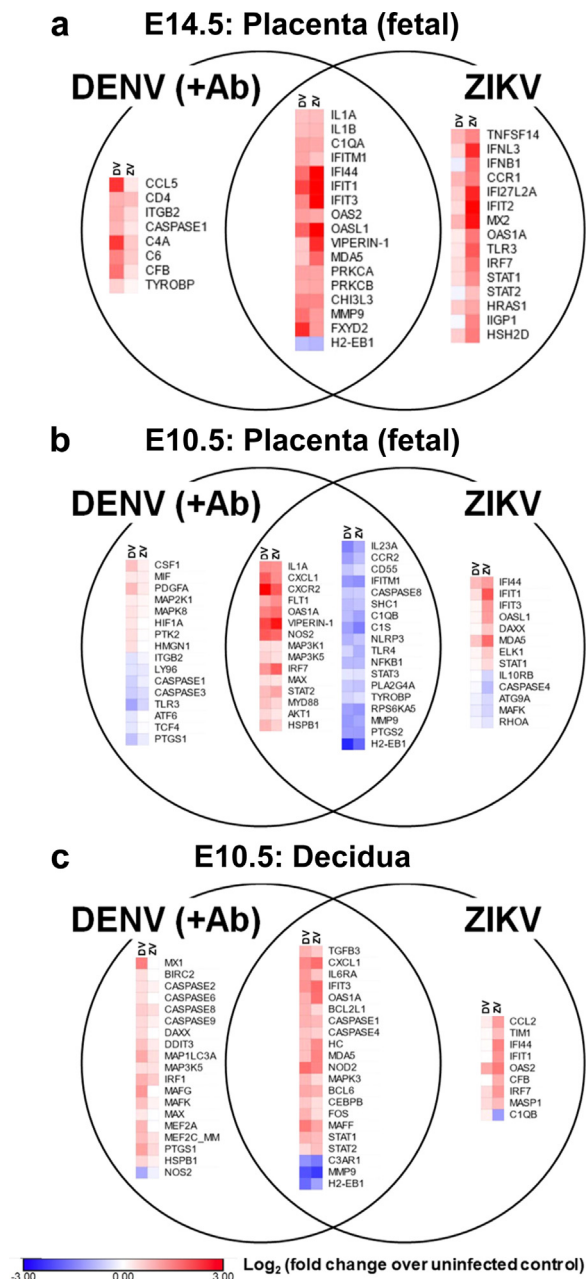

Figure 5. Selected genes that were significantly upregulated or downregulated by DENV and/or ZIKV infection. Gene counts for 278 endogenous genes were normalized to that of panel's internal housekeeping genes and expressed as log$_2$ (fold change) with respect to uninfected control for fetal placenta at E14.5 (Figure S5) and E10.5 (Figure S6), and decidua at E10.5 (Figure S7). The selected genes that were significantly upregulated or downregulated by DENV (+Ab) and/or ZIKV are shown with heatmap in the order of fetal placenta at E14.5 (a) and E10.5 (b), and decidua at E10.5 (c). Statistical analyses were performed using 2-tailed Student t-test and P value less than 0.05 was considered significant.
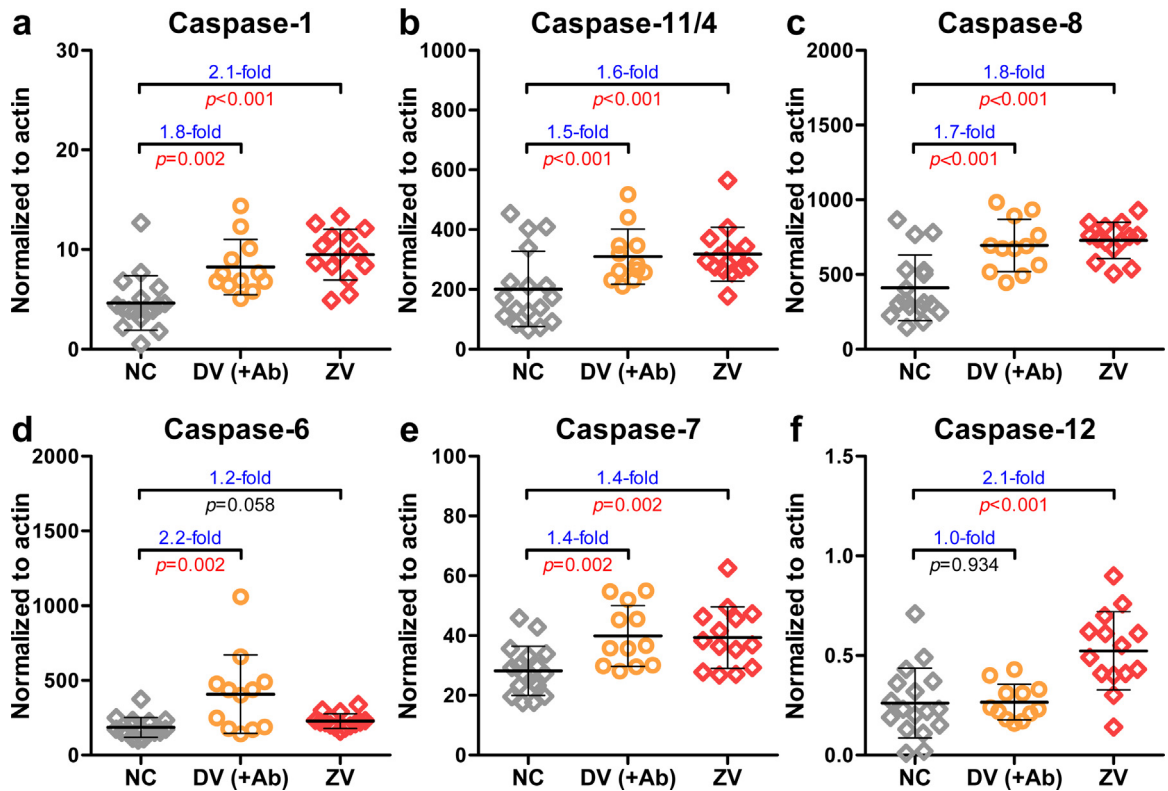
Figure 6. Modulation of caspase genes in decidua during DENV/ZIKV infection validated by real-time RT-PCR. RNA extracted from decidua samples of uninfected control ($n = 17$), DENV infection in the presence of Ab ($n = 12$) and ZIKV infection ($n = 14$) at E9−10.5 was subjected to real-time RT-PCR for Caspase-1 (a), Caspase-11/4 (b), Caspase-8 (c), Caspase-6 (d), Caspase-7 (e) and Caspase-12 (f). The gene expression was normalized to β-actin expression and presented as relative values calculated by $2^{-\Delta\Delta Ct}*10,000$. Statistical analyses were performed using 2-tailed Student t-test.

was specifically upregulated by DENV infection (Figure 6d) as observed by gene profiling (Figure 5c). Additionally, Caspase-7 (Figure 6e) and Caspase-12 (Figure 6f), which are not included in the nCounter® codeset, were found to be upregulated by DENV/ZIKV or only ZIKV infection, respectively. Taken together, these results indicate that DENV infection can evoke a broad range of host immune responses comparable to ZIKV despite lower placental viral burden in a mouse model.

Discussion

Viruses are thought to rarely cross the placental barrier, with particular exceptions such as Cytomegalovirus (CMV) and ZIKV that are known to infect the fetus, resulting in severe birth defects such as microcephaly or even fetal death.[61] Unfortunately, the mechanism of vertical transmission of viruses is not well understood and there are limited therapeutic or preventative strategies to block this transmission route. While mice infection models have various limitations, it is the most widely available tool, especially for ZIKV, to explore

maternal-fetal transmission with virus replication intact.[32] In this study, we demonstrated that DENV can also be transmitted vertically at a high rate in mice. Thus, this mouse model would be a novel avenue to investigate the mechanism of the transplacental virus infection.

It has been reported that adverse fetal outcomes correlate with the severity of maternal symptoms of dengue.[15,19] Since severe dengue severity is usually associated with secondary heterotypic infection, it is conceivable that the presence of enhancing cross-reactive antibodies increases the risk of fetal pathology in humans. Correspondingly, our results clearly showed that the rates of fetal DENV infection and death drastically increase in the presence of Ab that mimic the ADE condition concomitant with increased placental viral load. Although the high rate of fetal death (up to 50%) observed in our mouse model does not seem to truly reflect human fetal pathology, higher risk of miscarriage and stillbirth in pregnant women who had symptomatic dengue infection compared with those without should be noted.[10,15]

DENV pathogenesis in human placenta and fetus has not been thoroughly examined, probably due to a

lack of severe forms of fetal/infant abnormalities such as microcephaly. There is also lack of clinical information regarding the association of the rate of DENV vertical transmission with the serological status of dengue. Therefore, it is unclear whether the phenomenon found in our study that DENV fetal infection is accelerated in the presence of enhancing Abs is also applicable in the context of human infection. This is an unexplored area and our finding may open the door for more detailed investigation of the phenomenon. It is generally known that fetal placental portion comprises syncytiotrophoblasts and Hofbauer cells, both of which express Fc-gamma receptors,[62] while decidua comprises maternal macrophages which also express Fc-gamma receptors that are necessary to induce ADE.[57] Therefore, it is conceivable that these Fc-receptor-bearing cells are responsible for the induction of the high rate of fetal infection, and determining the DENV-infected cell types in the presence of enhancing Abs would be important to know the mechanisms in which ADE contributes to the enhancement of vertical transmission of virus in future studies. A recent study reported that ZIKV infection is enhanced in trophoblasts in the presence of DENV-immune serum both in mice and human placental explants,[59] suggesting that ZIKV pathogenesis in pregnancy is affected by the DENV-serological status. Thus, ZIKV infection under ADE condition would also be important to examine in order to understand the mechanism of vertical transmission of flaviviruses. However, the induction of ZIKV-ADE *in vivo* does not seem to be straightforward as DENV-ADE since our previous study showed that passive transfer of DENV-immune serum does not clearly induce ZIKV-ADE in non-pregnant A129 mice.[39] One of the interpretative limitations in our present study is that we used only one type of monoclonal Ab (4G2) to induce ADE, that has been used extensively in previous studies[35,39] and also shown to enhance ZIKV-induced fetal infection and demise in a different mouse model.[63] Use of polyclonal Abs is more relevant to clinical conditions and may elicit different responses than a single monoclonal Ab. Another limitation in the study is that there is no control mice receiving a non-specific monoclonal Ab. Some of these can be addressed in future studies.

We previously showed that ZIKV infection in pregnant A129 mice induces infection in 44% of the fetuses, and this vertical transmission was completely prevented by an antiviral treatment (nucleoside inhibitor) accompanied with 35-fold reduction in placental viral load.[34] Moreover, the rate of fetal infection was reduced from 48% to 14% by the 2-day delayed treatment with 3.4-fold reduction in placental viral load.[34] These results suggest that successful suppression of placental viral load by the antiviral treatment can prevent the transplacental virus infection. Interestingly, however, we found in the present study that the placental viral load is more than 20-fold lower in DENV infection under ADE

condition than ZIKV infection despite the comparable rate of fetal infection, suggesting that placental viral load is not only the determinant for the vertical virus transmission. The mechanism(s) that permit vertical transmission of viruses is unknown. Epidemiological studies showed that ZIKV infection during the first trimester results in higher severe congenital abnormalities and pregnancy complications than infection during the second and third trimesters.[51,64] Despite structural placental differences between mouse and human, the uterine tissue response to pregnancy is similar between the two species in terms of hemochorial placentation,[65] which may explain the reason why fetal ZIKV infection is most likely to occur at early stages of pregnancy in both humans and mice. Placental infection may lead to breakdown of the placental barrier by disrupting the endothelial junctions, as is thought to systemically occur during severe dengue infection,[66] resulting in ZIKV/DENV dissemination into the placenta. It has been reported that TNFα that is produced by virus-infected cells increases vascular permeability in tissues, resulting in shock syndrome and death in DENV mouse models.[35,55,67] Therefore, we initially hypothesized that TNFα-induced placental damage may allow the vertical virus transmission. However, a treatment with anti-TNFα Ab in ZIKV-infected pregnant mice could not protect fetal infection (unpublished data), suggesting that TNFα production in placenta is not responsible for vertical transmission of ZIKV in mice. Indeed, neither ZIKV nor DENV alters the expression of TNFα significantly in our gene profiling data.

Placenta undergoes dynamic changes that accompany fetal development from implantation to the parturition. In mice, decidualization and placentation with labyrinth formation begin around E6.5 and E8.5, respectively, and the structurally mature placenta is established around E10.5−11.5.[56,68] Thus, the vertical transmission of ZIKV and DENV occurs most effectively in the environment where extensive cell proliferation and remodeling are underway in the uterus. Interestingly, our gene expression profile in fetal placenta at E10.5 revealed that, unlike E14.5, many genes are downregulated by DENV and/or ZIKV infection that include apoptosis-related proteins and transcription factors such as NF-κβ. Therefore, it is conceivable that the altered gene expression caused by virus infection may interfere with the proper construction of placental barrier and consequently permit the transplacental transmission of virus.

It is also noteworthy that, although many genes are transcriptionally active in decidua at E10.5, they appear to be silenced and unresponsive to viral infections at the later stage (E13−14.5) in pregnancy. This decidual silencing seems to be associated with the placental development when it becomes functionally mature at E10.5−11.5.[56,68] Additionally, it may be relevant to the unique maternal-fetal interface environment where the

maternal immune system must be tolerant to fetal allo-antigens.[69] Nevertheless, to our knowledge, there is so far no report describing drastic changes in gene expression levels in decidua between early and late gestation stages in animal models as well as humans.[70] Intriguingly, a number of the apoptosis-related genes and transcription factors are upregulated predominantly by DENV infection at E10.5 in decidua. This may be associated with the condition of ADE since it has been shown that DENV infection in the presence of Ab induces caspase-dependent apoptosis in myeloid lineage cell line.[71] Morphologically, decidua does not directly contact the placental labyrinth zone where maternal and fetal blood flow in a counter-current direction across the spongiotrophoblast cells in the mature placenta.[56] However, in the primitive placenta at E8−10, the decidua occupies a large portion of placenta, surrounding the embryo across the trophoblast giant cells,[56] and is thought to regulate arterial remodeling and placental formation by producing several soluble factors such as cytokines.[68,72] Thus, the possibility that virus infection in decidua may modulate the decidual roles on the arterial remodeling and placentation that eventually influences on the equilibrium of the placental barrier cannot be excluded. More profound investigation in this area will be required to better understand the impact of virus infection on the process of placentation.

In conclusion, we demonstrated in an animal model that DENV can cross the placental barrier even though the level of placental infection is significantly lower than that of ZIKV infection. Detailed analysis of the molecular events associated with decidualization and placentation during DENV and ZIKV infection may serve to identify the key factor(s) responsible for vertical transmission of flaviviruses and develop potential preventative tools to block such transmission.

Data sharing statement
Data in this study is available upon request from the corresponding author at satoru.watanabe@duke-nus.edu.sg.

Contributors
S.W. and S.G.V conceived and designed the study. S.W., K.W.K.C., N.W.W.T., M.B.A.M., and A.C. performed the *in vivo* experiments and virological assays. S.W. and K.W.K.C. performed NanoString nCounter gene expression assay. K.T.F.C supported the interpretation of placental gene expression data. S.W and S.G.V accessed and verified the underlying data and were responsible for the decision to submit the manuscript. S.W. and S.G.V wrote the manuscript and all authors read and approved the final version of the manuscript.

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